

Formulation, Evaluation and HPTLC Analysis of Topical Ointment Containing *Calendula officinalis* and *Echinacae purpurae* Mother Tinctures

Sruthi R.¹, Biju C. R.², Jyotisree G.³, Amita S. Menon⁴, Ayswarya K.⁵ and Babu G.⁶

¹Department of Pharmaceutical Analysis, Devaki Amma Memorial College of Pharmacy, Chelembra, Malappuram, 673634, Kerala, INDIA

²Department of Pharmaceutical Analysis, Devaki Amma Memorial College of Pharmacy, Chelembra, Malappuram, 673634, Kerala, INDIA

³Department of Pharmaceutical Analysis, Devaki Amma Memorial College of Pharmacy, Chelembra, Malappuram, 673634, Kerala, INDIA

⁴Department of Pharmaceutical Analysis, Devaki Amma Memorial College of Pharmacy, Chelembra, Malappuram, 673634, Kerala, INDIA

⁵Department of Pharmaceutical Analysis, Devaki Amma Memorial College of Pharmacy, Chelembra, Malappuram, 673634, Kerala, INDIA

⁶Department of Pharmaceutical Analysis, Devaki Amma Memorial College of Pharmacy, Chelembra, Malappuram, 673634, Kerala, INDIA

³Corresponding Author: jyothi.g.marar@gmail.com

ABSTRACT

Skin morbidities are the major threat faced by the young adults of India. Various factors like climate, genetic factors, environmental factors, hygiene, dietary habits etc. Contribute to this ailments. There are both oral and topical medication for treating skin diseases. Antimicrobial agents in creams, ointments, lotions etc. Are used for the dermal treatments when bacterial and fungal attack is suspected or diagnosed. In this study, an ointment for treating skin morbidities was formulated using *calendula officinalis* and *echinacae purpurae* mother tinctures, evaluation and HPTLC profiling of the formulated ointment was performed with toluene: ethyl acetate: formic acid: methanol in the ratio 14:10:2:1 as mobile phase with standard procedures and scanned with camag TLC scanner 3 at 254 and 366nm. the HPTLC profiling of the simple ointment and mother tincture ointments showed 8 spots and 7 spots at 254 nm respectively.

Keywords- Mother tincture ointment, Quercetin, Simple ointment

I. INTRODUCTION

Mother tinctures are the 1/ 10 dilution of the crude extracts in certain specific amount of alcohol with minimal quantity of homeopathic preparation. Since these preparations are administered at even more diluted form they can neither be detected nor be quantified in the final product so, these preparations were considered to be free of the major safety concerns. Yet certain aspects cover the safety of these preparations, including the use of highly potent drugs- sometimes these mother tinctures are administered in its most concentrated form and also some of the preparations from microorganisms, moss, algae,

lichens, ferns, animal organs, secretions and cell lines may cause safety problems [1].

Calendula officinalis and *Echinacae purpurae* mother tinctures are two homeopathic remedies of botanical origin. *Calendula officinalis* mother tincture is prepared from the flowering tops whereas the later is prepared from the roots of *Echinacae purpurae*. *Calendula officinalis* mother tincture is an excellent remedy for the treatment of keloids, rheumatic pain, wounds and mouth ulcers. It also has good antiseptic, antimicrobial and diaphoretic property. *Echinacae purpurae* mother tincture is effective for the treatment of venom infections, purpural infections, cerebo-spinal meningitis. It also has immunostimulant and antimicrobial action [2-4].

Ointments are oily or greasy, homogenous, semisolid preparations intended for external application containing a hydrocarbon based ointment base. Ointment bases can be absorption, emulsifying, hydrocarbon, vegetable oil, and water soluble bases generally containing one or more medicaments in suspension or solution or dispersion. The activity of the preparation is mainly affected by the type of the base present in it. Longer action and emollient effect is obtained by combining hard paraffin, soft paraffin, wool fat and cetostearyl alcohol.

HPTLC can be considered as an important tool in routine drug analysis as it uses small quantity of mobile phase, reduces time and cost of analysis with minimum sample clean up. It also minimizes risk associated with exposure, disposal problems of toxic organic effluents, also facilitates repeatability of chromatograms with parameters. In addition to this there are no restrictions on the choice of solvents and mobile phases. Compared to TLC separation and resolution are

much better, and the results obtained are more reliable and reproducible. Hence HPTLC can serve as a tool for identification, authentication, and quality of herbal drug. The objectives of this work is to formulate and evaluate an ointment with the mother tinctures of *Calendula officinalis* and *Echinacae purpurae* with all scientific evidences and to analytically validate the formulated mother tincture ointment by HPTLC [5-7].

II. MATERIALS AND METHODS

Materials required

Wool fat (Fine chemicals), Hard paraffin (Fine chemicals), Cetostearyl alcohol (Fine chemicals), White soft paraffin (Fine chemicals), Rose oil, Toluene (E. Merck), ethyl acetate (E. Merck), formic acid (E. Merck), methanol (E. Merck), Quercetin (Fine chemicals)

Procurement of Mother tinctures

Mother tinctures of *Calendula officinalis* and *Echinacae purpurae* were purchased from Bakson Drug House Pvt. Ltd. and Dr. Wilmer Schwabe India Pvt. Ltd. respectively.

Preparation of stock solutions

10 mg of quercetin is accurately weighed and dissolved in methanol and water (1:1, v/v) to a make a stock solution of 1mg/ ml, which was diluted to 10 mL in standard volumetric flask. Working solution was prepared by mixing stock solution in such a way to produce a final concentration of 333.33ng/mL. This solution was finally filtered through a 0.45µm membrane filter before its application on TLC plate.

Preparation of simple ointment and mother tincture ointment

Table 1: Formula of Mother Tincture ointment

Ingredients	Quantity
Wool fat	5g
Hard paraffin	5g
Cetostearyl alcohol	5g
White soft paraffin	85g
Calendula officinalis	0.5ml
Echinacae purpurae	1.5ml
Rose oil	Q.S

A) Simple ointment preparation

Cetostearyl alcohol, white soft paraffin, wool fat was melted in a water bath in decreasing order of their melting points. To this melted hard paraffin was added upon constant stirring. Continue stirring until cold and attains an ointment consistency. Rose oil was added for fragrance.

B) Mother tincture ointment preparation

The simple ointment prepared was cooled and to this weighed quantities of mother tinctures are added and mixed well.

Evaluation of mother tincture ointment

a) Colour and Odour

Visual examination was used to study the colour and odour of the prepared ointment.

b) Consistency

By visual inspection consistency of the prepared ointment was tested.

c) Solubility

Solubility of ointment was checked in various inorganic and organic solvents.

d) pH

Digital pH meter was used to measure the pH. The solution of ointment was prepared by using 100ml of hot distilled water and kept aside for 2hrs. pH was determined in triplicate for the solution and average value was calculated.

e) Viscosity

Viscosity of prepared ointment was measured by using Broke field Viscometer.

f) Loss on drying

Petri-dish containing ointment was weighed and then it was placed on a water bath allowing it to dry at 105C. After drying the petri dish was reweighed again.

$$\% \text{ Loss on drying} = \frac{\text{Loss in weight of sample} \times 100}{\text{Weight of the sample}}$$

g) Spreadability

Excess of ointment was placed in between two slides which on to which uniform a definite weight was placed for a definite period of time. Spreadability is determined by the time required to separate the two slides. Lesser the time taken for separation of two slides better the spread ability.

Spread ability,

$$S = \frac{M \times L}{T}$$

Where,

S= Spread ability

M= Weight tide to the upper slide

L= Length of glass slide

T= Time taken to separate the slides

h) Extrude ability

The ointment is filled in a clean, lacquered aluminium collapsible tube and weighed. After that a pressure was applied on the tube with the help of fingers. Tube was reweighed again. Tube extrude ability was based on the amount of ointment extruded from the aluminium tube by application of pressure. More the quantity extruded better was the extrude ability [8-14].

HPTLC fingerprinting of simple ointment and mother tincture ointment 0.63 gram of ointment is extracted with 10ml methanol and the thin layer chromatography was performed by spotting 10µl of the simple ointment on Merk, 1.05554.0007, TLC silica gel 60 F254 20×10cm aluminium sheet using CAMAG linomat 5 spotter. The development is carried out using mobile phase Toluene: ethyl acetate: formic acid: methanol in the ratio 14:10:2:1 in CAMAG 20×10 cm twin trough chamber. Pre-saturation of the chromatographic chamber was performed for 30 min. Derivatisation was performed using iodine vapour and scanning was performed using CAMAGTLC scanner 3 at 254 and 366nm [15-17].

Validation of the formulated ointment

The proposed HPTLC method was validated as per the International Conference on Harmonization (ICH) guidelines Q2 (R1) for Accuracy, Linearity, Sensitivity, Robustness and Precision.

A) Accuracy

Recovery experiments was performed to evaluate the accuracy of the developed method using standard addition method. The known amount of quercetin was spiked to 3 different levels (80%, 100%, 120%) of a prequantified sample solution and percentage recovery was calculated from the response and the results were reanalysed thrice.

B) Precision

Intraday and inter day precisions are used for evaluating precision. Replicate analysis of freshly prepared standard solution of same concentration on the same day evaluates the intra- day precision whereas, this replicate analysis on different days gives inter- day precision.

C) Linearity and calibration curve

Linearity of the developed method was determined by using a calibration curve established with Asstandard concentration range from 100-300ng/spot. From the stock solution of quercetin in methanol, 10,20,30,40 µl were spotted on HPTLC plate so as to obtain the concentration of 100, 200, 300 and 400 ng/spot, respectively using automatic sample spotter. Each concentration peak area was plotted against the concentration of quercetin spotted and the r² value was calculated.

D) Limit of detection and quantification

Limit of detection and limit of quantification was used for determining the method sensitivity. Methanol (blank) was scanned six times to determine the signal to noise ratio. The calculation was based on the standard deviation (SD) of the response and the slope (S) of the calibration curve. The LOD was considered as 3:1 (SD/S) and LOQ as 10:1 (SD/S).

E) Robustness

The composition of mobile phase, amount of mobile phase, temperature, and duration of saturation was varied at a range ±10% and chromatogram was run to obtain the peak area from which standard deviation can be calculated [18-21].

III. RESULTS AND DISCUSSION



Figure 1: Formulation of Ointment

Table 2: Evaluation of mother tincture ointment

SI. No	Parameters	Inference
1.	Colour	White
2.	Odour	Characteristic

3.	Consistency	Soft Semisolid
4.	Solubility	Soluble in boiling water, miscible with alcohol, ether, chloroform
5.	pH	6.03
6.	Viscosity	14.303+-0.05
7.	Loss on drying	1.056%
8.	Spredability	6S
9.	Extrudability	Easily extrudable

The organoleptic and physicochemical parameters of the mother tincture ointment were studied and this shows satisfactory results for pH, spreadability, extrudability solubility, loss on drying and consistency HPTLC profiling of simple ointment and mother tincture ointment.

In simple ointment there are 8 spots with Rf value 0.13, 0.18, 0.32, 0.37, 0.41, 0.53, 0.61, 0.75 at 254nm. Of these spots in 10µl, 0.75 is predominant than

the other compounds. This is because all the other spots have relatively small percentage area. At 366nm, only 1 spot is observed with Rf value 0.37 and its percentage area was found to be 100%.Rf value of propylene is 0.13, this coincide with the Rf value of the spot in simple ointment. So one of the spot present in simple ointment is that of propylene. 1-Butene has the Rf value 0.17, which is approximately equal to that of the sp with Rf value 0.18.

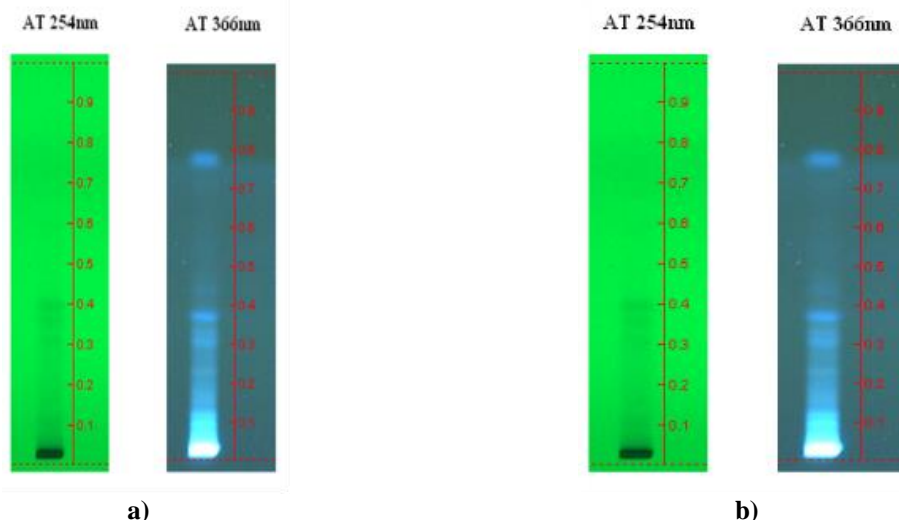


Figure 2: TLC Plate views of

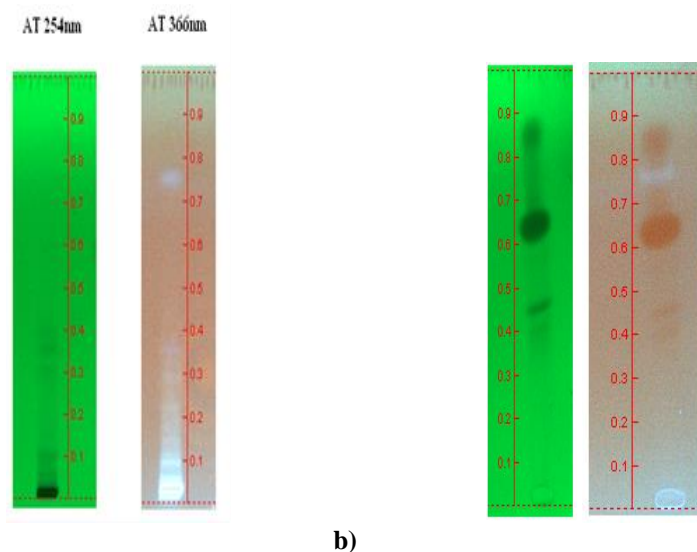


Figure 3: Derivatised TLC plate views of

Table 3: Rf Value & % Area of Ointment Sample at 254 nm

a) Simple ointment

Rf value	Area(AU)	% Area(AU)
0.13	452.3	2.78
0.18	265.7	1.63
0.32	1902.0	11.70
0.37	2304.7	14.19
0.41	2425.4	14.92
0.53	585.5	3.60
0.61	1439.2	8.85
0.75	6886.3	42.35

b) Mother tincture ointment

Rf value	Area(AU)	% Area(AU)
0.13	440.4	1.31
0.43	4084.9	12.19
0.47	6773.1	20.21
0.55	1447.5	4.32
0.60	2693.3	8.04
0.78	16748.9	49.98
0.93	1323	3.95

In *Calendula officinalis* and *Echinacae purpurae* ointment, there are 7 spots with Rf values 0.13, 0.43, 0.47, 0.55, 0.60, 0.78, 0.93 at 254 nm. Of these 0.78 is found to be predominant than the others as it has 49.98 of the percentage area. The Rf value 0.43 of the spot in ointment coincide with the Rf value of standard quercetin in *Calendula officinalis*. The range of Rf value of caffeic acid in *Calendula officinalis* is from 0.74 to 0.80 which corresponds to the Rf value 0.78 in the ointment. 0.62 is the Rf value of chlorgesic acid in *Echinacae purpurae* which corresponds to the fifth spot in the ointment. Also,

the fourth spot with Rf value comes within the range of Rf value of caftaric acid (0.51-0.57) in *Echinacae purpurae*. The third spot with Rf value 0.47 corresponds to that of alkaloids

Validation of the formulation

A) Accuracy

The degree of closeness of test results to true value depicts the accuracy of an analytical method. Determination of accuracy can be done by the application of analytical procedure to the recovery studies.

Table 4: Recovery Studies

Recovery Studies (n=3)				
Percentage of standard in sample	Theoretical Content(ng)	Amount of drug Recovered(ng± SD)	Percentage of drug recovered	%RSD
100	80	180	180.67±1.111	1.11
100	100	200	199.87±1.404	1.27
100	120	220	225.87±3.704	0.49

B) Precision

Table 5: Precision data of Quercetin

Concentration(nm)	Inter day precision		Intraday precision		Inter analyst precision	
	Mean peak area±SD	%RSD	Mean peak area±SD	%RSD	Mean peak area±SD	%RSD
133.33	1639.66±6.84	0.41	1638.77±4.49	0.27	1640.96±7.84	0.47
333.33	2715±7.07	0.26	2717.71±9.01	0.33	2716±7.87	0.28
666.66	4451.52±6.57	0.14	4452.92±7.51	0.16	4442.86±6.77	0.15

Three different concentrations with their replicates were prepared and these solutions were analyzed on the same day to record intra-day precision. In order to record the inter-day precision these standard solutions were analysed on three consecutive days.

C) Linearity and Calibration curve

The linear regression of standard curve was determined with $r^2 \pm SD = 0.9978 \pm 5.5$. The linear regression line is $y = 68.308x$. The regression data have shown a good linear relationship over the concentration range of 50-150 µg/ml. The SD for intercept value is noticed to be less than 2%.

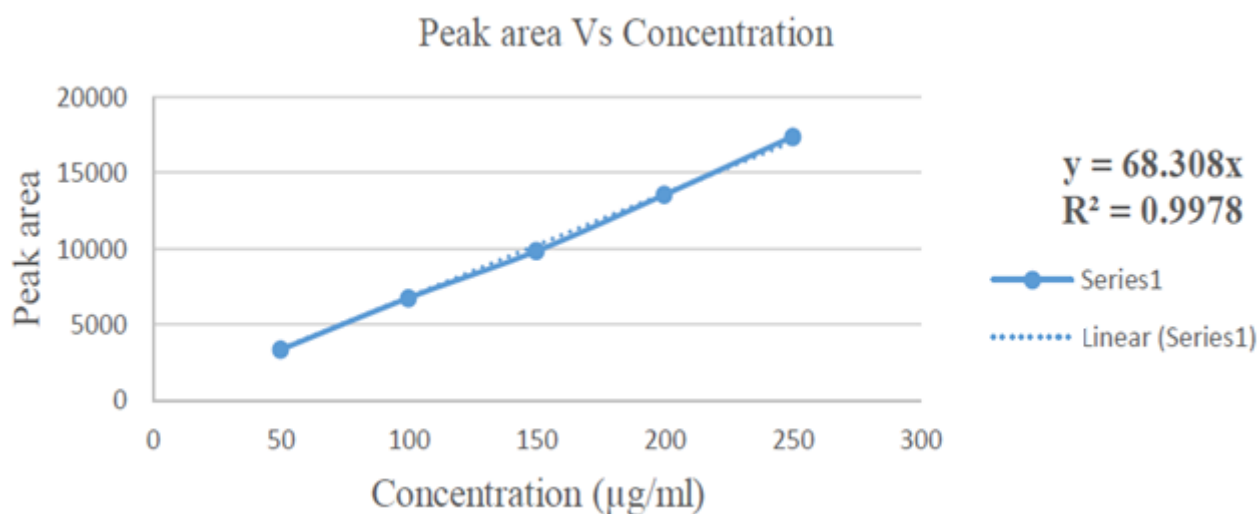


Figure 4: Linearity Plot of quercetin

D) Limit of detection and quantification

LOD and LOQ calculations were based on the standard deviation of y- intercept of the regression line (SD) and the slope (S) of the calibration curve, using $LOD = 3.3(SD/S)$, $LOQ = 10(SD/S)$ and LOD and LOQ were calculated as 225ng/ml and 700ng/ml.

E) Robustness

The standard deviations of peak areas were calculated for each parameter and %RSD was found to be less than 2%. The low values of % RSD obtained after introducing small deliberate changes in the developed HPTLC method, indicated the robustness of the method.

IV. DISCUSSION

Based on the allopathic system of medicine, an ointment was formulated with *Calendula officinalis* and *Echinacae purpurae* mother tinctures. The formulated ointment passes the physicochemical evaluation parameters. HPTLC profiling shows the presence of alkaloids, flavanoids and other phenolic compounds in the formulated mother tincture ointment. As these mother tinctures are herbal preparations and this herbal preparations being complex mixtures of various natural compounds, HPTLC profiling furnishes the therapeutic efficacy of the herbs. This HPTLC profiling can also be used for quality control of the formulated ointment. The developed method was also validated in terms of the ICH guidelines and was found to be accurate, sensitive and linear.

ACKNOWLEDGEMENT

The authors wish to thank the management, Devaki Amma Memorial College of Pharmacy, Malappuram, Kerala and Vaidyaratnam P.S. Varier's Arya Vaidya Sala, Kottakkal for providing the laboratory facilities.

REFERENCES

- [1] Safety issues in the preparation of homeopathic medicines. (2009). World Health Organisation.
- [2] WHO guidelines on good manufacturing practices (GMP) for herbal medicines. (2007). World Health Organization, Geneva.
- [3] Quality control methods for medicinal plant materials. (1998). Geneva, World Health Organization, 1998.
- [4] Homoeopathic pharmacopoeia of India. (1970) Delhi, *Controller of Publications*. 1(1), 22-28.
- [5] Carter SJ, Cooper and Gunn. (1987). *Dispensing for pharmaceutical students*. (12thed.). New Delhi: CBS Publishers.
- [6] A Banker GS, Rhodes CT. (1979). *Modern Pharmaceutics* (5thed.). Mumbai: Marcel Dekker
- [7] Remington. (1995). *The Science and practice of pharmacy*. (119thed.). New Delhi: Mack Publishing company.
- [8] Pattanayak, S., Nayak, S. S., Dinda, S. C., Panda, D., & Navale, K. P. (2011). Evaluation of Herbal Ointments Formulated with Methanolic Extract of *Cajanus scarabaeoides*. *Journal of Pharmacy and Allied Health Sciences*, 1(2), 49–57. <https://doi.org/10.3923/jpahs.2011.49.57>
- [9] Babae, N., Moslemi, D., Khalilpour, M., Vejdani, F., Moghadamnia, Y., Bijani, A., Baradaran, M., Kazemi, M. T., Khalilpour, A., Pouramir, M., & Moghadamnia, A. A. (2013). Antioxidant capacity of calendula officinalis flowers extract and prevention of radiation induced oropharyngeal mucositis in patients with head and neck cancers: a randomized controlled clinical study. *Daru : journal of Faculty of Pharmacy, Tehran University of Medical Sciences*, 21(1), 18. <https://doi.org/10.1186/2008-2231-21-18>
- [10] Patel, R. B., Patel, M. R., Bhatt, K. K., & Patel, B. G. (2011). Development and Validation of HPTLC

Method for Estimation of Carbamazepine in Formulations and Its *In Vitro* Release Study. *Chromatography Research International*, 2011, 1–8. <https://doi.org/10.4061/2011/684369>

[11] Gul, R., Jan, S. U., Faridullah, S., Sherani, S., & Jahan, N. (2017). Preliminary Phytochemical Screening, Quantitative Analysis of Alkaloids, and Antioxidant Activity of Crude Plant Extracts from *Ephedra intermedia* Indigenous to Balochistan. *The Scientific World Journal*, 2017, 1–7. <https://doi.org/10.1155/2017/5873648>

[12] Jyothi, D., Koland, M., & priya, S. (2014). Investigation of anti-inflammatory activity of ointments containing fenugreek extract. *Asian Journal of Pharmaceutical and Clinical Research*, 7(7), 66-69. <https://innovareacademics.in/journals/index.php/ajpcr/article/view/1029>

[13] Hassib, S. T., Mahrouse, M. A., Elkady, E. F., & Sayed, R. M. (2018). Simultaneous HPLC Determination of Betamethasone Esters-Containing Mixtures: Analysis of Their Topical Preparations. *Journal of Chromatographic Science*, 56(8), 716–723. <https://doi.org/10.1093/chromsci/bmy047>

[14] Archita J Patel. (2014). Development and Validation of Stability Indicating HPTLC Method for Simultaneous Estimation of Fluocinolone Acetonide and Miconazole Nitrate in Ointment. *Austin Chromatography*, 1(5), 1-9.

[15] Avbunudiogb, J. A., Enwa, F., Arhewoh, M. I., Builders, P. F., & Oni, A. C. (2014). Design and microbial screening of herbal ointment of *Phyllanthus amarus*. *International Journal of Phytopharmacy*, 4(4), 113-116.

<https://ssjournals.com/index.php/ijpp/article/view/1194>.

[16] Avish D Maru, & Swaroop R Lahoti. (2019). Formulation and evaluation of ointment containing sunflower wax. *Asian Journal of Pharmaceutical and Clinical Research*, 115–120. <https://doi.org/10.22159/ajpcr.2019.v12i18.33199>

[17] Fahimi, S., Abdollahi, M., Mortazavi, S. A., Hajimehdipoor, H., Abdolghaffari, A. H., & Rezvanfar, M. A. (2015). Wound Healing Activity of a Traditionally Used Poly Herbal Product in a Burn Wound Model in Rats. *Iranian Red Crescent Medical Journal*, 17(9). <https://doi.org/10.5812/ircmj.19960>

[18] R, B., & K, V. (2018). High-performance thin-layer chromatography fingerprint profile of *bauhinia tomentosa* linn. leaves. *Asian Journal of Pharmaceutical and Clinical Research*, 11(2), 344. <https://doi.org/10.22159/ajpcr.2018.v11i2.23294>

[19] Senguttuvan, J., & Subramaniam, P. (2016). HPTLC Fingerprints of Various Secondary Metabolites in the Traditional Medicinal Herb *Hypochoeris radicata* L. *Journal of Botany*, 2016, 1–11. <https://doi.org/10.1155/2016/5429625>

[20] Md. Sarfaraj Hussain, Sheeba Fareed, Mohammad Ali, Md. Sarfaraz Alam, Md. Akhlakquer Rahman, A. K. Srivastava, Jamuna Senguttuvan and Paulsamy

Subramaniam. (2014). Phytochemical investigation and simultaneous estimation of bioactive lupeol and stigmaterol in *Abutilon indicum* by validated HPTLC method. *Journal of Coastal Life Medicine*, 2(5), 394-401. <https://doi.org/10.12980/JCLM.2.2014J23>

[21] Mrudula B. Kulkarni, Pratibha B. Dange and Sanjay G. Walode. (2012). Stability indicating thin-layer chromatographic determination of chlorzoxazone, diclofenac sodium and paracetamol as bulk drug: Application to forced degradation study. *Der Pharmacia Sinica*, 3(6), 643-652.